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Transfection of Cultured Mesenchyme Cells Isolated from Embryonic Chick Limb Buds and Facial Primordia

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The bony structures of both the face and limb develop from outgrowths of undifferentiated mesenchyme cells encased in a single layer of ectoderm (10,11). Although the mesenchyme cells of limb buds and facial primordia have different embryonic origins that are derived from lateral plate mesoderm and neural crest, respectively (10,11), there is evidence that there are some similarities between the signaling pathways responsible for controlling their outgrowth and patterning. For example, the outgrowth of both limb buds and facial primordia requires reciprocal signaling between ectoderm and mesenchyme (6, 11). Tissue recombination experiments in the chick embryo have shown that the signals operating in the limb buds and facial primordia are interchangeable to some extent (7). Moreover, there are some similarities between the expression patterns and functions of signaling molecules such as bone morphogenetic proteins and fibroblast growth factors in developing chick limb buds and facial primordia (1,2,8).

One way to study the mechanisms by which gene expression is regulated in mesenchyme cells from embryonic chick limb buds and facial primordia is to identify DNA sequences that regulate the transcription of genes by performing transient reporter gene assays. Here, we report the establishment of conditions for the transient transfection of cultured dissociated mesenchyme cells isolated from chick embryo limb buds and facial primordia. We first examined whether cultured dissociated mesenchyme cells isolated from chick embryo limb buds or facial primordia could be transfected using a standard calcium phosphate coprecipitation technique.

Fertilized White Leghorn chicken eggs were obtained from Poyndon Farm, Waltham Cross, Hertsfordshire, England, UK, and incubated at 38°C $\pm$ 1°C. Stage 20 chick embryo wing buds

(3) were dissected into sterile PBS, placed in calcium- and magnesium-free Hank's buffered salt solution (Life Technologies, Paisley, Scotland, UK) containing 2% (wt/vol) trypsin (Life Technologies) and left on ice for 20–35 min to allow ectoderm and mesenchyme to separate. The solution was replaced with ice-cold culture medium (DMEM containing 10% [vol/vol] heat-inactivated fetal bovine serum, 2% [vol/vol] heat-inactivated chick serum, 200 mM L-glutamine and 100 U/mL of penicillin and streptomycin, all from Life Technologies, and the ectoderm was removed using fine forceps. The mesenchyme was placed in a sterile microcentrifuge tube, and cell suspensions were made by pipetting the tissue vigorously. The mesenchymal cells were pelleted by brief microcentrifugation, resuspended in culture medium and seeded at 6×10^4 cells per well in 24-well tissue culture plates.

Stage 23 chick embryo facial primordia (3) were dissected into sterile PBS, and cells were isolated and cultured as described by Wedden et al. (9). However, the cells were seeded at 6×10^4 cells per well in 24-well tissue culture dishes in Nutrient Mixture Ham's F-12TM supplemented with 200 mM L-glutamine, 10% (vol/vol) heat-inactivated horse serum and 100 U/mL penicillin and streptomycin (all from Life Technologies).

Both cell types were cultured at 37°C in a humidified incubator in an atmosphere containing 5% CO₂ until they reached 40% confluency. They were then transfected with pGL3-Promoter

plasmidTM (Promega, Southampton, England, UK), in which the luciferase reporter gene is under the control of the simian virus 40 (SV40) promoter, or with pGL3-Basic plasmid (Promega), in which there is no promoter.

Each reporter plasmid was transfected in triplicate. For each set of three wells of cells to be transfected, 5 µg reporter plasmid DNA were mixed with 37 µL 2 M CaCl₂ and adjusted to a final volume of 300 µL with deionized water. This mixture was added dropwise to 300 µL 2× HEPES-buffered saline (HBS), comprised of 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄·2H₂O, 12 mM dextrose and 50 mM HEPES, pH 7.05. Then, 200 µL of this mixture were added dropwise to each of the three wells. The cells were incubated for 6–8 h at 37°C in a humidified incubator in an atmosphere containing 5% CO₂, after which the cells were washed with serum-free medium and fresh complete medium added. After a further 48 h of culture, the cells were harvested and analyzed using a Luciferase Assay SystemTM (Promega) according to the manufacturer's instructions.

As shown in Figure 1, high levels of luciferase activity were detectable in facial primordia mesenchyme cells transfected with pGL3-Promoter as compared to the background in cells transfected with pGL3-Basic. In contrast, no luciferase activity was detected in limb bud mesenchyme cells transfected with pGL3-Promoter beyond the background detected in cells transfected with pGL3-Basic. We therefore assessed other methods for transfecting

Figure 1. Calcium phosphate co-precipitation-mediated transfection of pGL3-Basic (white bars) or pGL3-Promoter (black bars) into mesenchyme cells isolated from chick embryo limb buds (LB) or facial primordia (FP). Each value shown is the mean $\pm$ standard deviation for triplicate determinations.

Figure 2. Lipofectin-mediated transfection of pGL3-Basic (white bar) or pGL3-Promoter (black bar) into mesenchyme cells isolated from chick embryo limb buds. Each value shown is the mean $\pm$ standard deviation for triplicate determinations.

Benchmarks

limb bud mesenchyme cells. No luciferase activity above the background was detectable in limb bud mesenchyme cells transfected with pGL3-Promoter by electroporation (5) using a range of standard conditions (data not shown).

Lipofectin-mediated transfection was performed by adding 5 µg reporter plasmid to 500 µL optiMEM™ (Life Technologies) for each set of three wells of cells to be transfected. Lipofectin™ (Life Technologies) was added in a 1:4 ratio (DNA:Lipofectin) and complexes allowed to form for 10 min. The mixture was adjusted to 1500 µL with PBS, and 500 µL were added to each of the three wells. After 3 h at 37°C in a humidified incubator in an atmosphere containing 5% CO₂, the cells were washed in PBS and complete medium was added. Cells were cultured for a further 48 h, after which the medium was discarded and the cells harvested for reporter gene assays.

As shown in Figure 2, the use of Lipofectin resulted in high levels of lu-

ciferase activity above background in limb bud mesenchyme cells transfected with pGL3-Promoter. Lipofectin-mediated transfection of facial mesenchyme cells did not give significantly greater luciferase activity than calcium phosphate co-precipitation (data not shown).

In many cell types, the efficiency of Lipofectin-mediated transfection can be enhanced by adding an integrin-binding peptide to the DNA and Lipofectin mixture (4). We transfected limb bud mesenchyme cells with pGL3-Promoter using a range of such Lipofectin/Integrin-binding peptide/DNA (LID) reagents, but none of them enhanced the level of luciferase activity obtained with Lipofectin alone (data not shown).

These results show that mesenchyme cells isolated from chick embryo facial primordia can be readily transfected using a standard calcium phosphate co-precipitation technique. However, the transfection of mesenchyme cells isolated from chick embryo limb buds requires the more costly Lipofectin technique. The reason for this difference in behavior is unclear.

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Fast, Qualitative Analysis of p53 Phosphorylation by Protein Kinases

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The function of the tumor suppressor p53 is regulated by multiple phosphorylations that are induced by different stress signals and cooperate in modulating p53 response (3). Therefore, it is important to define precisely which kinases phosphorylate p53 and where their phosphorylation sites are